tometric procedures to within an average of 5% (CS) or 10% (CPT) and with the NADH fluorometric procedure for CS to within an average of 1% (Table 2). Precision, as indicated by the coefficient of variation (CV) for sample replicates, averaged 4.4% for the CS ABD-F assays and 1.7 and 2.3% for NADH fluorometric and spectrophotometric assays, respectively. CVs for CPT are 6% for both procedures and are similar to those reported for an alternative fluorometric assay (14).

*Scaling down ABD-F assay.* If necessary, assay volumes may be scaled down. When assays are conducted on samples with relatively high enzymatic activities (as was the case for CS in the present work), the protein precipitation step and subsequent centrifugation may be omitted (i.e., the small contribution of protein thiols to blank fluorescence may be tolerated). In such cases, the assay may be adapted for high-throughput microwell plates with reagent volumes reduced appropriately. For example, in measuring CS activity in individual copepods (Class Crustacea) weighing approximately 100–200 μg dry weight, we have modified the protocol to conduct the assay in a 96-well microplate using 100 μl reagent medium and 10 μl homogenate (one copepod in 500 μl homogenizing medium). The reaction is stopped with the addition of 20 μl SSA (17.5%), neutralized with 50 μl of $KHCO_3$ (1 M) before labeling with 20 μl of ABD-F (2 mg $ml^{-1}$). $KHCO_3$ is added slowly to avoid vigorous bubbling as $CO_2$ is generated.

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# Detection of PCR Products in Real Time Using Light-up Probes

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Real-time PCR,[2] where the amount of product is measured during ongoing amplification, is the most sensitive method to determine the amount of a specific DNA in a sample. Product is detected in homogeneous solution, by the binding of a fluorescent reporter. During the past few years a number of technologies for homogeneous probing, both nonspecific (1–3) and specific (4–6), have been developed. In the simplest assay the PCR product is detected through the binding of double-strand DNA specific dyes, such as the asymmetric cyanine dye SYBR Green I (3, 7). These kinds of dyes have essentially no fluorescence of their own and become intensely fluorescent when they bind to nucleic

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[2] *Abbreviations used:* PCR, polymerase chain reaction; FRET, fluorescence resonance energy transfer; PNA, peptide nucleic acid; BSA, bovine serum albumin; ntc, no template control; TO, thiazole orange.

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acids (8). A problem when using free dyes is that they do not recognize a specific product and bind to any products formed such as various primer–dimer products. To distinguish different products, post-PCR fluorescence-mediated DNA melting curve analysis is required (9). Accordingly, real-time PCR using free dyes is not suitable for multiplexing. Further, free dyes bind to any genomic DNA, which requires the DNA of interest to be purified before PCR analysis. To overcome these drawbacks specific probes have been developed. These are based on various oligonucleotide–dye conjugates that hybridize to an internal sequence of the amplified product. Most of these probes, e.g., TaqMan (4), Molecular Beacons (5), and the Hybridization probes (10) are based on two dyes, a fluorophore–acceptor pair, that are engaged in fluorescence resonance energy transfer (FRET). Usually the acceptor dye acts as quencher.

Recently, we presented a novel reporter, the light-up probe (Fig. 1), for sequence specific detection of target DNA in homogeneous solution (11). The light-up probe is a peptide nucleic acid (PNA) (12, 13) to which an asymmetric cyanine dye is tethered. Upon probe hybridization the dye binds to target DNA, which results in large enhancement in dye fluorescence. The light-up probe design confers several advantages. Using a single reporter dye, it is sufficient to register the increase in fluorescence intensity, instead of measuring the change in the fluorescence intensity distribution necessary when using energy transfer probes. Another advantage is that the light-up probe becomes luminescent upon regular hybridization and can readily be designed to bind at annealing temperature and dissociate during elongation. Hence, it does not interfere with the PCR process as, for example, the TaqMan probe, which is degraded by the polymerase during the extension phase; a process that also makes regular three-step PCR more complicated. Nor is a probe conformational change, such as in Molecular Beacons, required to generate signal upon hybridization. Further, being based on an uncharged analog (PNA), the light-up probe hybridizes faster and binds target DNA much stronger than oligonucleotide-based probes (13, 14) and successfully competes with reannealing of heat-denatured template. These advantages make it very simple to develop light-up probed real-time PCR assays starting from standard protocols.

We have previously shown that light-up probes hybridize to single stranded target DNA with high sequence discrimination and with up to 50-fold enhancement in fluorescence (11). We have also shown that light-up probes do not interfere with the PCR reaction and that they identify specific products in post-PCR assays with high sensitivity (15). Here we demonstrate that light-up probes are suitable also for real-time PCR applications using, as example, the *gusA* gene (*Escherichia coli* β-glucuronidase gene), which is a common reporter gene in transgenic plant research (16). A segment of the *gusA* gene is amplified in the presence of a *gusA*-specific light-up probe and the amount of product is monitored in real time by the probe fluorescence signal.

## *Materials and Methods*

*Light-up probe.* The light-up probe gusLUP was synthesized by coupling *N*-carboxypentyl-4-[(3′-methyl-1′,3′-benzothiazol-2′-yl) methylenyl] quinolinium iodide to PNA with the sequence H-TTCTTTTTCC-lys$^+$-$NH_2$ as described previously (11). The probe was purified by reversed phase HPLC to remove free dye, which would give rise to nonspecific signal, and shorter PNAs (11). Integrity of stored probes was checked before use by HPLC. Molar absorptivity of the probe at 260 nm was estimated as the sum of the contributions from PNA (17) and TO: $\epsilon_{260} = 11{,}700n_C + 6600n_C + 13{,}700n_A + 8600n_T + \epsilon_{260}^{TO}$ (=7100 $M^{-1}$ $cm^{-1}$ (18)) = 87,100 $M^{-1}$ $cm^{-1}$. The concentration of the probe was determined by absorption measurement at high temperature (85°C), where the PNA has lost all secondary structure (17). The thermal melting temperature ($T_m$) of the probe–target complex was estimated by determining the $T_m$ for a probe–oligonucleotide complex by $A_{260}$ measurements (in 10 mM Tris, pH 8.3, 30 mM KCl, 5 mM $MgCl_2$) (11). The oligonucleotide (Medprobe) had the sequence 5′-AGGTACT**GGAAAAA-GAA**CTTCTGGC-3′, where the target sequence is indicated with bold letters.

*Real-time PCR.* A 1098-bp DNA segment of the *gusA* reporter gene (1.9 kb) cloned in the plasmid pJIT166 (19) was amplified by PCR using the primers 5′-AACTATGC-CGGAATCCATCG-3′ and 5′-ACATATCCAGCCATGCA-CAC-3′. The amplified product contained the sequence 5′-(955 bases)...GGAAAAAGAA...(133 bases)-3′, targeted by the gusLUP probe in antiparallel orientation. PCR (20 μl) samples contained 1 × PCR buffer (Sigma), 4 mM $MgCl_2$, 600 μM of each dNTP (Sigma), 2 U *Taq* polymerase (Sigma), 0.6 μM of each primer (Medprobe), 4 μg BSA (MBI Fermenta), and 0.4 μM gusLUP. The rather high concentrations of *Taq* polymerase and BSA used are due to extensive adsorption to the large surface area of the LightCycler glass capillaries (20). The PCR reaction was "hot-started" by low-temperature inactivation of the *Taq* polymerase by premixing it with the TaqStart antibody (0.44 μg/2 U *Taq*) (Clontech). A plasmid dilution series was prepared from a stock solution containing 0.01 μg/μl by sequential dilution in poly(dA) solution (20 μg/μl). Real-time PCR was measured in a LightCycler (Roche Diagnostics) using the thermocycler program: 3 min preincubation at 95°C followed by 40 cycles of 0 s at 95°C, 20 s at 54°C, and 40 s at 74°C. Fluorescence was monitored at the end of the annealing phase using 470 nm excitation and 530 nm emission (F1

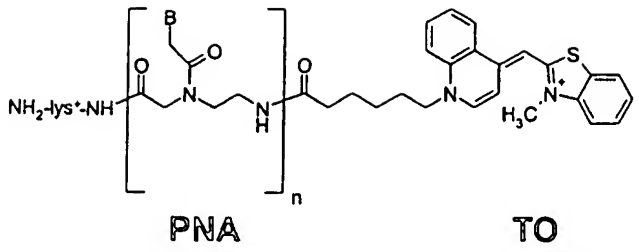


FIG. 1. Structure of the gusLUP probe (B denotes nucleobases). The asymmetric cyanine dye thiazole orange (TO) is conjugated to a PNA with the sequence lys$^+$-CCTTTTTCTT.

channel). After completed PCR the samples were analyzed by agarose gel electrophoresis to verify product formation.

*Results and Discussion*

A 1098-bp fragment of the *gusA* reporter gene (16) containing a sequence targeted by the light-up probe gusLUP (Fig. 1) was PCR amplified in the presence of gusLUP in a real-time PCR instrument (LightCycler, Roche Diagnostics). gusLUP was designed to have a probe-target thermal melting temperature ($T_m$ = 67°C) in between the primer annealing temperature (54°C) and the elongation temperature (74°C) in order to bind at annealing and dissociate during the PCR extension phase. Hence, the fluorescence of the hybridized gusLUP recorded at annealing temperature reflected the amount of PCR product formed during the reaction. In a PCR reaction the number of cycles needed to obtain a particular amount of product is negatively proportional to the logarithm of the number of template molecules originally present in the sample. When analyzing real-time PCR data a threshold fluorescence signal level is chosen that is significantly above the background noise, and the number of cycles required to reach this level is determined (2).

Figure 2 shows real-time PCR amplification of a dilution series of the *gusA* plasmid. A stock solution containing 0.01 μg/μl plasmid was diluted to give samples containing $10^9$, $3.2 \times 10^8$, $10^8$, . . . , $3.2 \times 10^2$ copies ($3.2 = \sqrt{10}$). A "no template control" (ntc) was included for comparison and all reactions were performed in duplicates. Figure 2a shows fluorescence intensity recorded at 530 nm as a function of the number of PCR cycles ('amplification plot'). The threshold was set to 30 times the standard deviation of the background calculated from the readings in the first five cycles.[3] After eight cycles significant fluorescence increase was observed for the sample containing $10^9$ copies of plasmid, and the threshold was crossed at 8.85 cycles. As the PCR reaction advances the signal continues to grow far above the threshold. The second sample in the dilution series reached the threshold after 10.5 cycles. This is 10.5 − 8.85 = 1.65 cycles later.

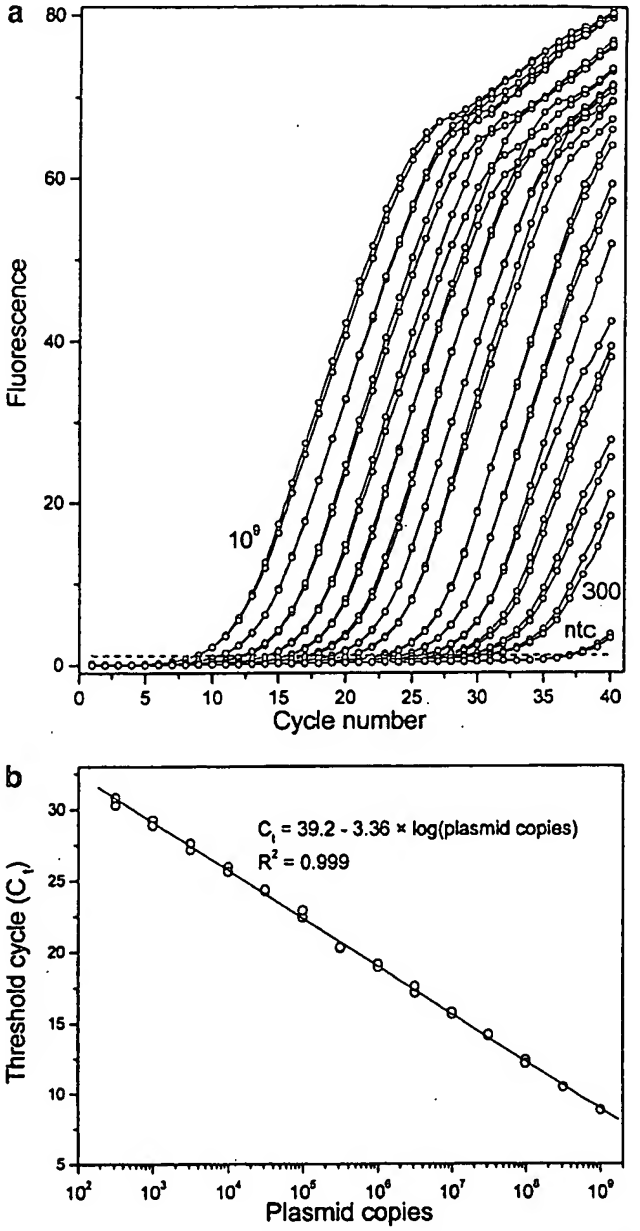


FIG. 2. (a) Amplification plot (o) of a dilution series from $10^9$ to 300 copies in steps of 3.2 ($=\sqrt{10}$) of plasmid. Duplicate samples. A "no template control" (ntc) was included for comparison. The background signal estimated by the average intensity in the first five readings in each reaction was subtracted. The dashed line indicates the threshold level. (b) The threshold cycle ($C_t$) as function of the initial number of plasmid copies (logarithmic scale) (o), and linear regression (—) of $C_t$ vs log (plasmid copies). Slope = −3.36, $R^2$ = 0.999.

[3] The threshold was set significantly above the noise-level according to the recommendations in the LightCycler Software Manual.

which corresponds to $2^{1.65} \approx 3$ times difference in the amount of starting material assuming 100% PCR efficiency. Figure 2b shows a plot of the number of cycles needed to reach the threshold level ($C_t$) vs the logarithm of the number of initial plasmid copies. The plot is perfectly linear ($R^2 = 0.999$) with a slope of −3.36. This is in accordance with exponential growth with 100% efficiency (−log 10/log 2 ≈ −3.32). The average variation in the number of cycles at threshold for duplicate samples (Fig. 2), $\langle|C_t^2 - C_t^1|\rangle$, was 0.25 cycles corresponding to a difference in the estimated number of initial molecules ($N_1/N_2$) of less than 20% ($N_1/N_2 = 2^{\langle|C_t^2-C_t^1|\rangle} = 1.19$). Actually, for the five most concentrated samples ($10^9$–$10^7$ initial copies) the average variation was only 0.11 cycles corresponding to a difference in $N$ of 8% ($N_1/N_2 = 1.08$). After some 37 cycles a small signal develops in the ntc. We are presently uncertain why. It should not be due to contaminating free dye, since no signal is obtained when purified probe is added to noncomplementary oligonucleotides (11). It should not be due to any probe degradation products either, since HPLC analyses of probes run in the LightCycler revealed no degradation. It could be due to formation of a particular primer–dimer product that interacts with the probe. It could also be due to template contamination; the ntc $C_t$ corresponds to four initial template molecules. We are presently investigating the origin of the ntc signal.

In conclusion, we have shown that light-up probes are suitable for monitoring the amount of accumulated PCR product in real-time using a standard three-step PCR protocol. The reaction proceeds with 100% efficiency, evidencing that the light-up probe does not interfere with the PCR. The assay produces correct results in the range of $10^9$ to 300 template copies, suggesting that it is only limited by sample handling. The reproducibility is excellent, the difference in the estimated number of molecules being less than 20% over the range $10^9$ to 300 copies.

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